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AUTHORITY

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REPORT NO. 276

STUDIES ON THE ANTIPROTEOLYTIC ACTIVITY OF BOVINE BLOOD*

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Report No. 276
Project No. 6-64-12-028
Subtask USAMRL S-11
MEDEA

ABSTRACT

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To determine whether the antiproteolytic activity of bovine blood against trypsin and plasmin is due to one inhibitor or to separate proteolytic inhibitors.

RESULTS AND CONCLUSIONS

Antiproteolytic assays for bovine serum gave an average value of 11 ± 2 for the ratio of trypsin inhibiting to plasmin inhibiting activity. Ammonium sulfate fractionation, electrophoresis and heat inactivation were employed in an attempt to separate antitryptic and antiplasmic activity. However, no significant changes from the above antitrypsin/antiplasmin ratio were observed with the different inhibitor preparations. The results obtained, therefore, are in agreement with the assumption that the antitryptic and antiplasmic activity of bovine blood is due to a single inhibitor.

RECOMMENDATIONS

Further purification of the antiproteolytic preparations, obtained by electrophoresis of certain bovine plasma fractions, is indicated. These studies should be extended to human blood.

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STUDIES ON THE ANTIPROTEOLYTIC ACTIVITY OF BOVINE BLOOD

I. INTRODUCTION

It has been known for some time that mammalian blood inhibits trypsin and plasmin. The problem of specificity of this antiproteolytic activity is, at present, still not resolved. Several investigators (1-5) have presented evidence for the presence in human blood of different inhibitory agents, which vary in their specific effect on trypsin and on plasmin.

The present work is concerned with a comparison of the antitryptic and antiplasmic activities of various bovine plasma fractions using casein as a substrate. The ratio of antitryptic to antiplasmic activity for the various inhibitor fractions prepared was used as an index of whether or not all of the antiproteolytic activity of beef blood against trypsin and plasmin is due to one inhibitor or to separate proteolytic inhibitors. The data obtained are in agreement with the assumption that the antiproteolytic activity of beef blood against trypsin and plasmin is exerted by a singular inhibitory factor.

II. EXPERIMENTAL

A. Preparation of Various Antiproteolytic Fractions from Bovine Plasma.

The procedure for obtaining inhibitor fractions I and II is similar to that employed by Loomis and his associates (6) and by Peanasky and Laskowski (7).

1. Inhibitor Fraction I. Oxalated bovine blood was collected at the slaughter house, and centrifuged at 4° C. To one liter of oxalated plasma, cooled to 0-5° C, was added 1500 ml of 0.9% saline. After thorough mixing, 605 gm of ammonium sulfate was slowly added with stirring and the solution was allowed to stand at 0-5° C for at least 4 hours. After centrifugation in the cold for 45 min at 1300 g, the supernatant was adjusted to pH 3.7 to 3.8 with 5 N sulfuric acid. After stirring for 10 min at 4° C, the solution was centrifuged at 4° C for 45 min at 1300 g, the precipitate was discarded and the supernatant immediately adjusted to pH 6 to 7 with 5 N sodium hydroxide. To each 100 ml of solution 28.05 gm of ammonium sulfate was slowly added with constant stirring. After standing for about 4 hours in the refrigerator the solution was centrifuged. The supernatant was discarded and

the precipitate dissolved in a minimum of distilled water and dialyzed against distilled water at 4° C. After dialysis, the protein concentration of the solution was adjusted to 1% by the addition of distilled water and 42 gm of ammonium sulfate was added with stirring, at room temperature, for each 100 ml of solution. After standing at room temperature overnight, the solution was centrifuged. To the supernatant, 7 gm of ammonium sulfate was added for each 100 ml of the original 1% protein solution. After standing for 4 to 6 hours at room temperature, the solution was centrifuged and the obtained precipitate dissolved in a minimum amount of distilled water. After dialysis against distilled water at 4° C, the solution was lyophilized (inhibitor fraction I).

2. Inhibitor Fraction II. This preparation was obtained by further fractionation of inhibitor fraction I with ammonium sulfate. Inhibitor fraction I was dissolved in 0.9% saline at a protein concentration of 1%. For each 100 ml of the solution 35 gm of ammonium sulfate was added with stirring at room temperature. Any precipitate formed was removed by centrifugation, and the supernatant after cooling to 0° C adjusted to pH 4.0. After stirring for 10 min at 0° C, the solution was centrifuged in the cold and the supernatant adjusted to pH 7.0. Seven gm of ammonium sulfate were added for each 100 ml of the initial solution and the mixture left standing at room temperature for 4 to 6 hours. Any resulting precipitate was removed by centrifugation and 14 gm of ammonium sulfate for each 100 ml of the initial solution was added to the supernatant. The entire procedure was repeated three times or until no precipitate was formed except in the final fraction precipitated between 60 to 80% ammonium sulfate saturation. The final 0.4-0.8 SAS precipitate was dissolved in distilled water, dialyzed against distilled water at 4° C and lyophilized (inhibitor fraction II).

The various intermediate fractions obtained by fractionation of plasma by ammonium sulfate precipitation were found to exert none or only slight antiproteolytic activity per mg protein when compared to the final inhibitor fractions I and II.

B Electrophoretic Separation of Inhibitor Fraction II.

A continuous paper electrophoresis technique, as described by Selden and Westphal (8), was employed for further fractionation of inhibitor fraction II. The preparation was dissolved in Michaelis buffer (pH 8.6, μ = 0.05) at a protein concentration of about 6%. The solution was fed onto Whatman 3 MM paper for 24 hours at an applied potential 225v. The stained electrophoresis pattern showed essentially 4 fractions of different mobilities

C. Heat Inactivation Studies

Inhibitor fraction I or II in concentrations of 1.2% in Tham-NaCl buffer were heated at varying temperatures and length of time, as indicated in Tables 3 to 5, cooled rapidly in ice water and tested subsequently. For antiproteolytic assays, the solution was diluted with Tham-NaCl buffer to a concentration optimal for the determination of the antiproteolytic activity. In all cases, the antiproteolytic activity of the heated material was compared with that of an unheated control sample.

D. Enzyme Preparations

1. Trypsin

A stock solution was prepared which contained 3 to 4 mg of crystalline salt free trypsin* in 100 ml of 0.0025 N hydrochloric acid.

2. Plasmin

Two hundred gm of dried human plasma fraction III** were finely ground and extracted with 4 liters of 0.2 N sulfuric acid for half an hour at room temperature (9). About 10 gm of Celite was added to the mixture which was filtered rapidly through a Buchner funnel. The filtrate was immediately adjusted to pH 7 to 7.5 with 4 N sodium hydroxide and ammonium sulfate was added slowly with stirring to 20% saturation (114 gm of salt for each 100 ml of solution). The solution was filtered with the aid of Celite through a Buchner funnel, and the same amount of ammonium sulfate as used in the first precipitation was added to the filtrate. The mixture was allowed to stand at 4° C for 3 to 4 hours and the precipitate, obtained after centrifugation at 4° C for 1 hour at 1300 g, was dissolved in 300 ml of M/15 phosphate buffer (pH 7.2). To the solution were added 3000 units of streptokinase*** for each gm of fraction III used as starting material. The mixture was allowed to stand for 15 to 30 min at 25 to 26° C, adjusted to pH 3.9 with 1 N hydrochloric acid and dialyzed against N/1000 hydrochloric acid at 4° C until free of sulfate ions. The crude plasmin, obtained after

*Obtained from Worthington Biochemical Sales Company, Freehold, New Jersey.

**Obtained through the courtesy of the American National Red Cross and kindly supplied to us by the Sutter Laboratories.

***Streptokinase-Streptodornase Varidase was supplied to us through the generosity of Lederle Laboratories.

lyophilization, was made up to a 1% solution in N/1000 hydrochloric acid. Sodium chloride was added to a concentration of 3% and the mixture kept at 4° C for 3 to 4 hours. Any precipitate formed was removed by centrifugation and the supernatant was brought to 20% sodium chloride concentration. After standing for 2 hours at 4° C, the precipitate was removed by centrifugation, dissolved in N/1000 hydrochloric acid, dialyzed against N/1000 hydrochloric acid in the cold, and then lyophilized. The partially purified plasmin preparation was quite soluble in distilled water. It was found that the proteolytic activity of this plasmin preparation was apparently of a similar order as the human plasmin preparation* of Fishman and Kline (10), who employed a somewhat different preparation procedure.

E. Casein Substrate

A 1% casein (11) solution was prepared by heating 1 gm of casein in 100 ml of pH 7.4 phosphate-saline buffer in a water bath for 15 min. After filtration from traces of insoluble material, the solution was stored in a refrigerator. A fresh solution was made every 3 weeks.

D. Assay Procedure

1. Antifibrinolytic Test

In earlier experiments, attempts were made to determine also the antifibrinolytic activity of inhibitor fractions for trypsin and human plasmin. It was observed, however, in agreement with Sherry and his associates (12, 13), that the determination of fibrinolytic activity of streptokinase activated human plasminogen, using bovine fibrinogen and thrombin, may give erroneous results. This lack of reliability of the test was found, in agreement with Sherry and his associates (12, 13), to be due to possible contamination of the bovine fibrinogen and thrombin with plasminogen which is converted to plasmin by the streptokinase activated human plasmin. As a result of these findings, the antifibrinolytic assay was, at present, discontinued and the results on the antifibrinolytic activity of various inhibitor fractions thus far obtained have been omitted from this presentation.

*Kindly supplied to us by Dr. David L. Kline, Yale Medical School, New Haven, Connecticut.

2. Antiproteolytic Test

A modification of the methods of Kunitz (14) and of Remmert and Cohen (15) was employed for determining proteolytic and antiproteolytic activities using casein as substrate.

3. Reagents

TCA: A 5% solution of trichloroacetic acid (Mallinckrodt-USP Grade) was made in distilled water.

THAM-NaCl Buffer: 12.5 gm of tris(hydroxymethyl) aminomethane was dissolved in 500 ml of distilled water. After addition of 20 gm of sodium chloride and 85 ml of 1 N hydrochloric acid, the final volume was brought to 2 liters with distilled water and the pH adjusted to 7.25. All inhibitor preparations were dissolved in this buffer.

a. Determination of Antiproteolytic Activity. To 1 ml of buffered inhibitor solution was added 1 ml of standardized plasmin or trypsin solution*. Inhibitor and enzyme were permitted to react for 30 min at 25 to 26° C; 2 ml of 1% casein solution was then added and the mixture incubated for 30 min at 35° C ± 1°. As blanks suitable controls were set up. Enzyme activity was stopped in the blanks immediately after the addition of the casein solution and in the samples after 30 min incubation at 35° C ± 1° by the addition of 5 ml of 5% trichloroacetic acid. The tubes were allowed to stand at room temperature for 2 hours with occasional shaking. The precipitate was then removed by filtration through S & S #589 filter paper. The optical density of the TCA filtrates was read at 280 mμ against appropriate blanks in a Beckman DU Spectrophotometer. All antiproteolytic activity determinations were carried out in duplicate or triplicate.

b. Definition of Proteolytic and Antiproteolytic Activity. Ten trypsin or ten plasmin units were arbitrarily defined as that degree of proteolytic activity which would produce an optical density of 0.500 after incubation with casein for 30 min at 35° C ± 1°. It was found that 68 γ of the crystalline trypsin used corresponded to ten proteolytic trypsin units. Correspondingly, one antitrypsin or one antiplasmin

*It was found that solutions containing approximately 30 γ of trypsin or 2 mg of plasmin per ml were most suitable for these antiproteolytic studies.

unit would be equal to that amount of the inhibitor preparation capable of inhibiting 1 unit of trypsin (6.8 γ) or of plasmin respectively. Respective curves, obtained by plotting optical density against the enzyme units, were used to calculate the antiproteolytic activity of an inhibitor preparation for trypsin and for plasmin respectively (Fig. 1 and 2). The best reproducible and most reliable determinations were found to fall in the straight line part of the curves.

In the assay, the difference between the number of proteolytic units in the standard enzyme solution and in the enzyme solution containing the inhibitor is indicative of the number of enzyme units inhibited. The antiproteolytic activity of an inhibitor fraction has been expressed as antitrypsin and antiplasmin units per mg of preparation and formed the basis for the calculation of the ratio of antitrypsin to antiplasmin activity

III. RESULTS AND INTERPRETATION

The average value for the antitrypsin/antiplasmin ratio for the various preparations reported and several others not included in this presentation was found to be 11 ± 2. As can be seen from Table 1, the ratio between antitryptic and antiplasmin activity was 11.1 for beef serum. Several investigators (16, 17, 18) have already reported that the inhibition of plasmin requires a higher concentration of serum or plasma than is necessary to inhibit trypsin of equal proteolytic potency.

Should any separation of the antitrypsin and antiplasmin activity, present in beef blood, occur during the various stages of purification or heat inactivation, one would expect a corresponding change in the antitrypsin/antiplasmin ratio as established for beef serum. Inhibitor fractions I and II, obtained by ammonium sulfate fractionation of bovine plasma, gave an antitrypsin/antiplasmin ratio of 9.9 and 12.8 respectively, falling within the range of the antitrypsin/antiplasmin ratio established for bovine serum.

Table 2 presents the results obtained by electrophoretic separation. Most of the antiproteolytic activity was obviously concentrated in the fast moving component. The antitryptic activity, found for fractions D and E, would indicate that 1 mg of these inhibitor preparations would inactivate about 0.5 mg of crystalline trypsin. The data of Table 2 also illustrate that no significant change in the antitrypsin/antiplasmin ratio was found for any of the fractions obtained by electrophoresis.

Tables 3 through 5 present the results of heat inactivation experiments with inhibitor fractions I and II. The data show that the

antitrypsin/antiplasmin ratios for the various heated preparations held reasonably constant, exhibiting only minor variations well within the range of experimental error. It would appear, therefore, that the thermal destruction of antitryptic and antiplasmin activities occurred at the same rate. It seems that the more purified inhibitor fraction II is somewhat more heat stable than inhibitor fraction I (Tables 4 and 5).

Shulman (2) reported that heating of human serum at 60° C for 20 min decreased antitryptic activity to 10% of its original value while the ability to inhibit plasmin remained practically unchanged. As can be seen from the data of Table 3, comparatively little loss in antitryptic activity of inhibitor fractions I and II occurred on heating at 60° C for 45 min. It is quite possible that human blood may contain a heat labile specific antitrypsin inhibitor which is, apparently, not present in bovine blood. Recently Shulman (19) described an antitrypsin preparation from human plasma which is comparatively heat stable. The possible identity of this inhibitor with the antiproteolytic factor from bovine blood has still to be elucidated.

IV. DISCUSSION

The present findings are in agreement with the assumption that the antiproteolytic activity of bovine blood, measured against trypsin and plasmin, is due to a single inhibitor. The results are in contrast to the evidence for the presence in human blood of different proteolytic inhibitors, which differ in their specific effect on trypsin and plasmin (1, 2, 3, 4, 5). It is obvious from the present data that it was necessary to use approximately 10 times as much inhibitor preparation to inactivate the same proteolytic plasmin activity as trypsin activity. It may, therefore, be possible to observe mainly antitryptic and comparatively little antiplasmin activity if the amounts of the inhibitor preparation employed in the antiplasmin assay are not in the range of proper concentration. This ratio of antiplasmin to antitrypsin activity would have to be taken into account if one measures the antiplasmin activity of an inhibitor preparation.

V. SUMMARY

Experiments to separate antitrypsin and antiplasmin activity of bovine blood by ammonium sulfate fractionation, by electrophoresis, and by heat inactivation indicate that the antiproteolytic activity of bovine blood against trypsin and plasmin is due to a single inhibitory factor.

VI. RECOMMENDATIONS

Further purification of the antiproteolytic preparations, obtained by electrophoresis of certain bovine plasma fractions, is indicated. These studies should be extended to human blood.

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TABLE 1

ANTIPROTEOLYTIC ACTIVITY OF BOVINE SERUM AND OF INHIBITOR FRACTIONS I AND II

	Beef Serum Per ml	Inhibitor Fraction I Per mg	Inhibitor Fraction II Per mg
AP* Units	28	1.4	2.4
AT** Units	310	13.3	31.0
AT/AP Ratio	11.1	9.9	12.8

Average values of 4 experiments

*AP = Antiplasmin
**AT = Antitrypsin

TABLE 2

ANTIPROTEOLYTIC ACTIVITY OF ELECTROPHORESIS FRACTIONS

	Starting Material	Fraction A	Fraction B	Fraction C	Fraction D	Fraction E
AP Units/mg	2.3	0.5	1.0	1.6	5.6	7.2
AT Units/mg	28.0	5.5	12.0	18.0	74.0	79.0
AT/AP Ratio	12.0	11.0	12.0	12.0	13.0	11.0

Average of two separate experiments. Fractions A, B, and C refer to the slowly moving components collected. Fractions D and E are the fast moving components. The antiproteolytic activity of the slow as well as the fastest moving components was negligible and, therefore, is not recorded.

TABLE 3

HEAT INACTIVATION OF ANTIPROTEOLYTIC ACTIVITY AT 60° C FOR 45 MIN.

Material	Inhibitor Fraction I Unheated	Inhibitor Fraction I Heated	Inhibitor Fraction II Unheated	Inhibitor Fraction II Heated
AP Units/mg	1.6	1.2	2.5	2.2
AT Units/mg	14.0	11.0	32.0	29.0
AT/AP Ratio	9.0	9.2	12.8	10.5

Average values of 4 experiments

TABLE 4

HEAT INACTIVATION OF ANTIPROTEOLYTIC ACTIVITY AT 65° C

Material	Inhibitor Fraction I Unheated	Inhibitor Fraction I Heated, 30 Min	Inhibitor Fraction I Heated, 45 Min	Inhibitor Fraction II Unheated	Inhibitor Fraction II Heated, 45 Min
AP Units/mg	1.4	0.7	0.7	1.3	2.2
AT Units/mg	14.5	8.8	8.3	28.0	28.0
AT/AP Ratio	10.4	12.6	11.7	12.6	12.7

Average values of 4 experiments

TABLE 5

HEAT INACTIVATION OF ANTIPROTEOLYTIC ACTIVITY AT 70° C

Material	Inhibitor Fraction I Unheated	Inhibitor Fraction I Heated, 20 Min	Inhibitor Fraction I Heated, 30 Min	Inhibitor Fraction II Unheated	Inhibitor Fraction II Heated, 20 Min	Inhibitor Fraction II Heated 30 Min
AP Units/mg	1.5	0.3	0.2	2.5	1.3	1.5
AT Units/mg	12.5	4.0	2.5	32.0	18.0	13.0
AT/AP Ratio	8.3	11.8	10.4	12.8	13.8	10.8

Average of 4 experiments.

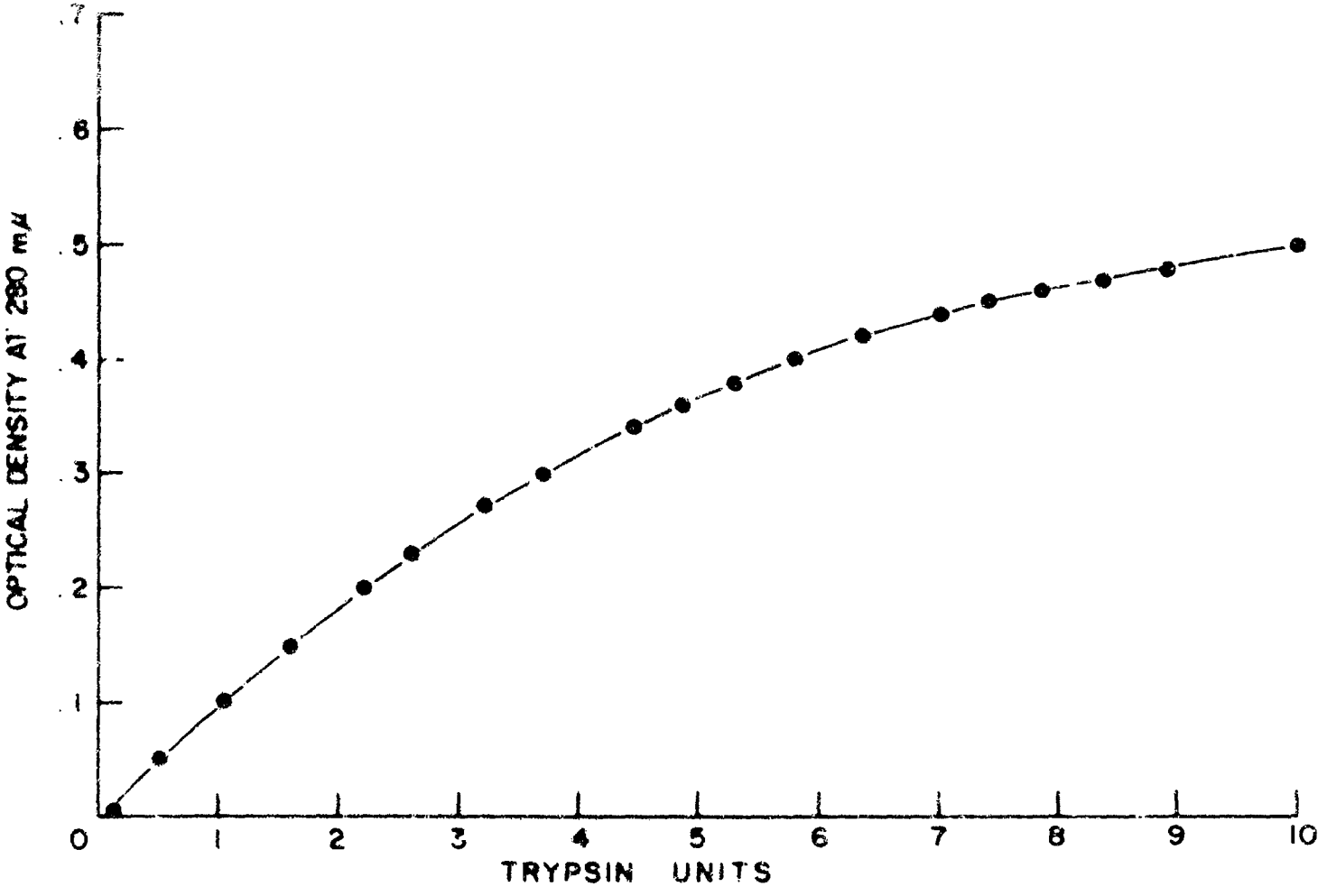

Fig. 1. Standard curve for the determination of trypsin units.

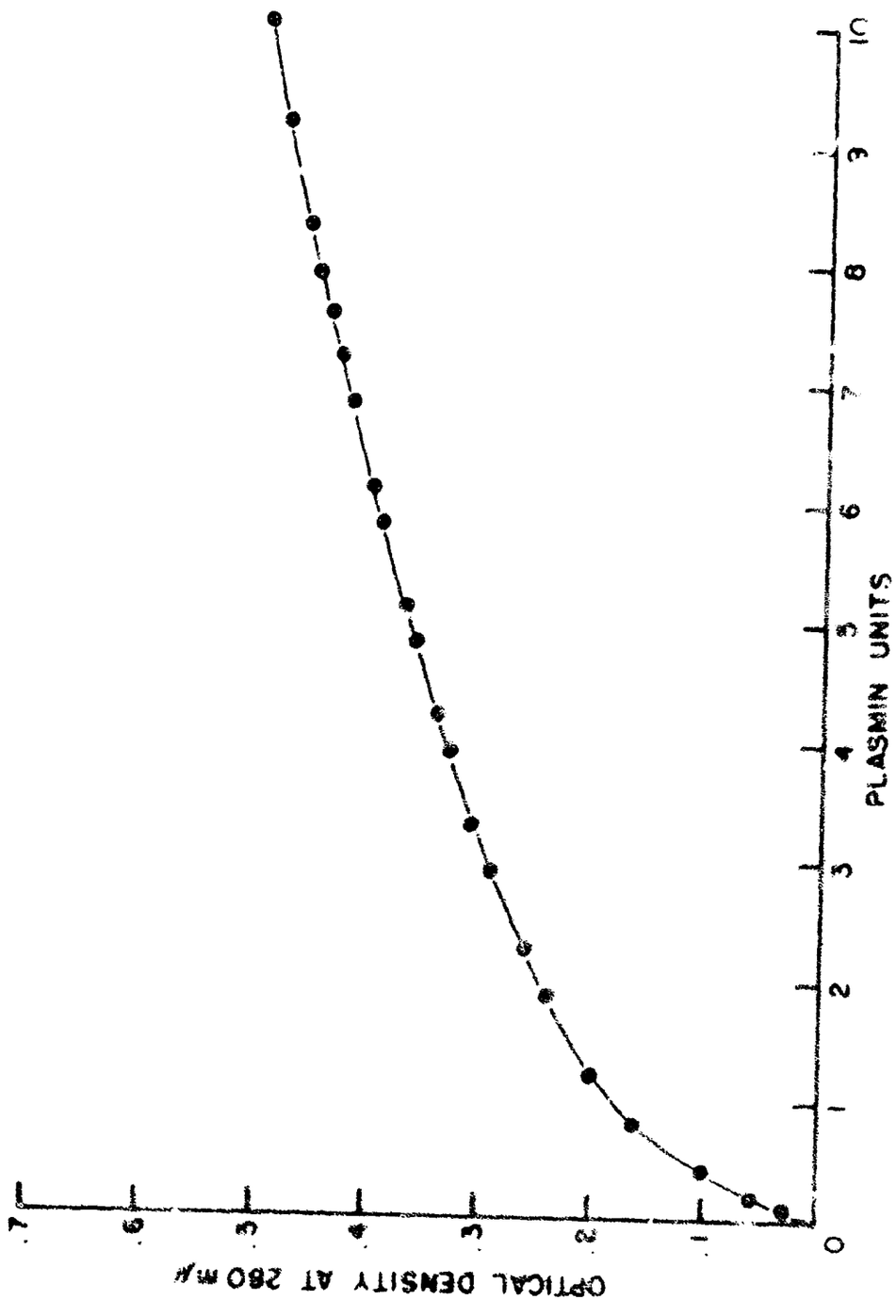

Fig. 2. Standard curve for the determination of plasmin units.

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